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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/00 — A2

(11) International Publication Number: WO 98/42824

(43) International Publication Date: 1 October 1998 (01.10.98)

(21) International Application Number: PCT/GB98/00654

(22) International Filing Date: 19 March 1998 (19.03.98)

(30) Priority Data:
9705744.2 — 20 March 1997 (20.03.97) — GB

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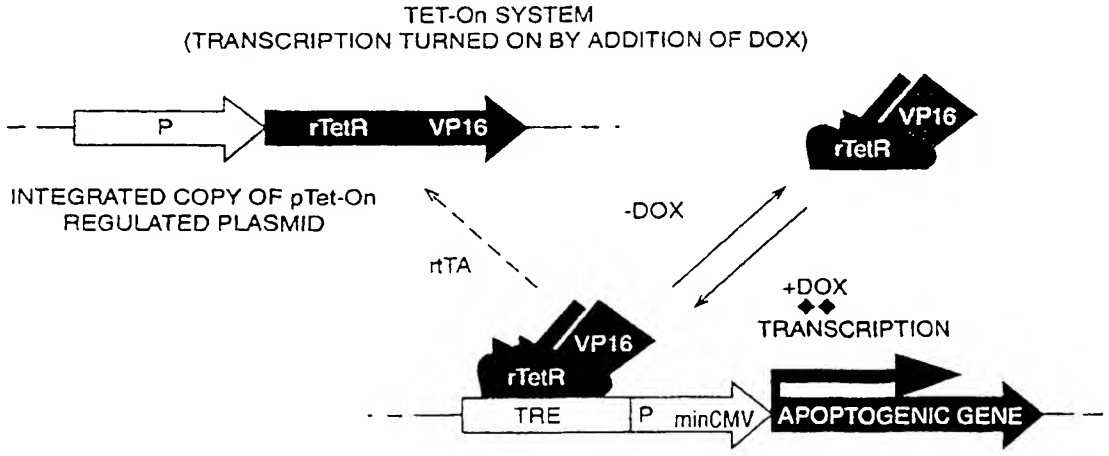
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: METHODS FOR SELECTING CELLS AND THEIR USES



(57) Abstract

Grafts, cells and tissues for use in transplantation, transgenic animals, methods of cell selection and various uses of such material.

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METHODS FOR SELECTING CELLS AND THEIR USES

The present invention relates to grafts, cells and tissues for use in transplantatio, to transgenic animals, methods of cell selection and to various uses of such material. In particular, the invention relates to cellular material derived from various organisms including humans, non-humans and transgenic organisms containing negative selectable marker can be induced to activate programmed cell death or apoptosis, e.g. for use in transplantation therapy.

It is known in the art that cells can be routinely engineered or induced to express gene(s) which confer any of a wide variety of selectable phenotypes thereon. Such genes are known as selectable markers. They are normally introduced into cells as part of a recombinant expression vector. The selectable phenotype conferred by a selectable marker may be classed as either positive or negative.

A positive selectable phenotype is one which permits survival under particular conditions which would kill (or at least prevent or impair the growth of) cells which do not exhibit the positive selectable phenotype.

A negative selectable phenotype is one which results in the destruction (or the prevention or impairment of growth) of the cell under particular conditions which are relatively innocuous to cells which do not exhibit the negative selectable phenotype. A negative selectable phenotype may be one which arises from the cell's own programmed cell death mechanisms, eg. the process of apoptosis.

A wide variety of selectable markers are available. Genes that are commonly applied as positive selectable markers include the bacterial neomycin phosphotransferase (neo; Colbere-Garapin et al. (1981) 150:1), hygromycin phosphotransferase (hph; Santerre et al. (1984) Gene 30:147) and xanthine-guanine phosphoribosyl transferase (gpt; Mulligan and Berg (1981) P.N.A.S. 78:2072).

Also used as positive selectable markers are the Herpes simplex virus type 1 thymidine kinase (HSV-1 TK; Wigler et al. (1977) Cell 11.223), adenine phosphoribosyl-transferase (APRT, Wigler et al. (1979) P.N.A.S. 76:1373) and hypoxanthine phosphoribosyltransferase (HPRT, Jolly et al. (1983) P.N.A.S. 80:477). These latter markers must be used in cells having a particular mutant genotype (viz. one which leads to a deficiency in the gene product on which the selection is based).

Some of the aforementioned genes also confer negative as well as positive selectable phenotypes. They include the HSV-1 TK, APRT, HPRT and gpt genes. These genes encode enzymes which can catalyse the conversion of certain nucleoside or purine analogues to cytotoxic intermediates. For example, the nucleoside analogue ganciclovir (GCV) is a good substrate for the HSV-1 thymidine kinase but a poor substrate for the natural thymidine kinase found in mammalian cells. Consequently, GCV can be used for efficient negative selection against mammalian cells expressing the HSV-1 TK gene (St. Clair et al. (1987) Antimicrob. Agents Chemotherap. 31 844). Xanthineguanine phosphoribosyl transferase can be used for both positive and negative selection when expressed in wild type cells (Besnard et al. (1987) Mol. Cell. Biol. 7 4139)

Selectable markers are usually used in both prokaryotic and eukaryotic genetic engineering to

permit the recovery from a mixed population of cells which have undergone a relatively rare genetic change. For example, they can be used in physical association with another gene which encodes a product of interest (for example, a therapeutic protein) to select cells which have taken up that other gene along with the selectable marker. For example, the neo gene has been used to monitor genetically modified cells taken from patient samples after gene therapy has taken place.

It has also been proposed to use negative selectable markers as a safety device in gene therapy. Many gene therapies involve the removal of somatic cells from the patient, the introduction therein of a therapeutic gene (the expression of which repairs a biochemical lesion), followed by reintroduction of the cells back into the patient. Since the reintroduced genetically modified cells may ultimately prove deleterious to the health of the patient (for example, if they prove to be immunologically incompatible or become malignant), a negative selectable marker may be introduced along with the therapeutic gene to permit (if necessary) subsequent selective elimination of the genetically modified cells.

A problem with the currently available negative selectable markers is the so-called bystander effect. For example, a cell which has been genetically engineered to express the viral thymidine kinase will convert GCV into a cytotoxic metabolite. Similarly, cells expressing cytosine deaminase will convert fluorocytosine (FC) to a cytotoxic intermediate also. The intracellular build-up of these cytotoxic metabolites leads to the death of the engineered cell. But, in addition, death of non-engineered cells close to the engineered cell can also occur. This is because cytotoxic intermediate(s) leak from the dying engineered cell into the local milieu, to be accumulated by the neighbouring non-engineered cells, resulting in some cases in their own death.

A second problem associated with the use of many of the currently available negative selectable markers is their inefficient killing non-dividing cells under certain circumstances. It is well known that the cytotoxic effect of the intermediate generated by viral thymidine kinase from (e.g. GCV) is mediated by interfering with the cell's DNA synthetic pathway. If the HSV-1 thymidine kinase-engineered cell is not undergoing DNA synthesis, then GCV and its cytotoxic intermediate do not so effectively elicit cell death. Similarly, only those cytosine deaminase expressing cells which are also replicative are most easily killed by FC administration.

Finally, the dosing regimen of known negative selectable agents such as GCV or FC in vivo needs to be of at least 10 days duration, and probably longer, in order to ensure complete eradication of all cells. In a clinical situation, where total compliance with this regimen might need to be ensured and therefore closely monitored, such a duration of treatment is often undesirable.

The invention mitigates these problems by providing inter alia methods for cell and gene therapy which permit the removal (if required) of engineered cells without a bystander effect, without the requirement that such engineered cells should be replicative, and with the possibility of a much reduced duration of administration of drugs to elicit the cytotoxic effect. The method is particularly advantageous for neural cell transplantation and gene therapy in the central and peripheral nervous systems (e.g. in the treatment of Parkinson's disease, Huntington's chorea, Alzheimer's disease, amyotrophic lateral sclerosis, ischaemia-induced and trauma-induced cell loss), where a pruning or titration, or even a complete elimination of the grafted cells may become necessary, but without compromising residual function amongst the host cells. Thus, such a selection method limits bystander effects during the removal of

the transplanted cells from the nervous system, and thereby attenuates damage to otherwise normal host tissue. In addition, when the grafted or genetically engineered cells are non-dividing cells then the negative selection of the invention (which does not rely wholly or partly on cell division for its effects) is particularly advantageous. Finally, a more accurate pruning of cells numbers might be achievable if the drug required to elicit the cell death needs to be given over a short duration.

The negative selectable markers of the invention exploit the natural mechanisms by which cells are depleted in vivo, for example during normal development. These mechanisms are collectively known as programmed cell death, and are thought to underlie the process of apoptosis. Apoptosis is a fundamental mechanism for regulating cell numbers at all stages of life in multicellular organisms. During embryogenesis, for instance, various cells are selected for apoptosis: this leads to the manifestation of biological form, via either phylogenetic or morphogenetic shaping. Programmed cell death also occurs in the normal adult. For example the vertebrate haematopoietic system undergoes huge cell losses, with billions of neutrophils dying this way each day. Apoptosis also plays a major role in tissue repair and regeneration: when a cell is damaged beyond repair by e.g. ultra-violet irradiation, cell death is triggered using the cell's own suicide programme. It is thought that this altruistic act helps to maintain the organism as a whole by guarding against further replication of damaged cells which may become cancerous, thereby leading to the death of the whole organism. The control of apoptosis is currently a major component of worldwide research in cancer, since it is now believed that some cancers may result, not from an enhanced replication of tumour cells, but from a reduced ability of such cells to undergo programmed cell death.

Another advantage of using a cell's natural mechanism of programmed cell death is that macrophages and microglia react to apoptotic cells in a highly specific fashion. They engulf and digest apoptotic cells, but do not secrete inflammation-inducing signals, in contrast to their secretory profile when phagocytosing necrotic cells. Cells undergoing apoptosis rather than toxic cell death and necrosis, therefore, are not associated with a localized inflammatory response - a second unwanted contributor to the bystander effect of currently used negative selection markers.

To activate the cell disposal mechanism, apoptotic cells change their surface chemistry so that macrophages/microglia can recognize them. The alteration may be in the surface lectins or in integrins, and may vary depending on the cell type, but the final death is still via apoptosis.

The invention rests at least in part on the recognition that the natural cellular mechanism of apoptosis can be used as a safety and/or titration switch to eliminate genetically engineered cells and, in particular, cells transplanted to the central nervous system. Using such a mechanism, it is possible to induce the apoptosis and subsequent elimination of grafted cells (e.g. in the brain). This method is used advantageously, because:

such grafted cells may not necessarily be replicative, (an at least partial requirement for ablation by negative selectable markers such as HSV-1 thymidine kinase or cytosine deaminase.

the grafted cells can be removed without the bystander effect of HSV-1 thymidine kinase due, for example, to leaching of cytotoxic intermediates from the dying cells, and the localized inflammatory response they provoke. Such a bystander effect could be particularly

detrimental in the central nervous system.

Another advantage is that apoptosis may be induced by a relatively short duration of treatment compared to the duration of treatment required to produce non-apoptotic cell death. Experiments indicate that e.g. up-regulation of biologically active p53 may induce apoptotic cell death within hours: by contrast, the cytosine deaminase or HSV-1 thymidine kinase negative selection mechanisms currently used require many days of treatment.

Any negative selectable marker which can trigger programmed cell death may be used in the present invention. For example, several factors are currently thought to be involved in the signalling and mediation of apoptosis. In particular, apoptosis can result from increased expression of p53, stimulation of the cell surface Fas receptor, or activation of the interleukin-1b converting enzyme family of cysteine proteases. In addition, c-myc, retinoblastoma, and the bax-bcl2 and related genes are involved in apoptosis. These are the subject of reviews (Lin et al. (1995) Canc. Surv. 25:173; Timmers & Kremer (1995) Biomed. Rev. 4:103; Hale et al. (1996) Eur. J. Biochem. 236:1; Fraser et al. (1996) Curr. Op. Neurobiol. 6:71; Shimamura & Fisher (1996) Clin. Can. Res. 2:435; Vaux & Strasser (1996) P.N.A.S. 93:2239; Spence et al. (1996) Exp. Op. Therap. Pat. 6.345; Gilardini et al. (1996) Clin. Immunotherap. 5 413) which are incorporated as support. In particular, we and others (Slack et al. (1996) J. Cell Biol. 135:1085) have found overexpression of the tumor suppressor gene p53 to be of particular advantage in inducing the apoptosis of neural cells. Any of the foregoing factors may be used as the basis of the negative selectable markers according to the invention.

Transgenic organisms bearing a selectable marker as a transgene are known in the art and are described, for example, in a pending patent application (PCT GB95 02586, WO 96/14401) which is incorporated by reference herein. These transgenic organisms may provide tissue and cells for transplantation, as well as having other uses. Furthermore, a number of vectors bearing positive or negative selectable markers have been made and are readily available to those skilled in the art (for review see Miller (1992), Nature 357:455). Others may be readily assembled using standard gene cloning techniques.

Another important problem arises from the need for cultures of a single tissue or cell type. Growth in vitro from single cells may be difficult (often requiring the use of feeder cells and/or mixtures of growth factors and other supplements) and homogeneous in vitro populations cannot therefore be easily obtained. Homogeneous populations which also comprise a negative selection marker where programmed cell death can be induced are particularly difficult to produce.

There is therefore a need for a convenient source of selectable cells/tissues of all types for primary culture or other purposes such as tissue transplantation and cell/gene therapy

It has now been found that transgenic organisms bearing a negative selectable marker which induces programmed cell death have previously unrecognized utility in cell culture techniques, and tissue transplantation and cell/gene therapy

The present invention provides for, but is not limited to, transgenic organisms which inter alia constitute a very convenient source of material for the isolation, identification, culture and analysis of cells from any tissue of the organism's body. Tissue dissected from the transgenic organisms of the invention can be particularly easily grown (even as homogeneous populations of a particular cell/tissue type) in vitro and used in a wide variety of applications,

including pharmaceutical assays, tissue transplantation, cell/gene therapy, general factor synthesis, drug delivery and protein production.

For reasons of clarity, transgene means the genetic structure that is transferred to the cell. Although the term transgenic organisms has occasionally been applied to any organism which contains foreign DNA, the term "transgenic organism" is used herein in its more usual sense to denote eukaryotic organisms (and in particular, animals or plants, and especially vertebrates e.g. mammals) and their progeny which contain heterologous chromosomal DNA in the germ line. The heterologous chromosomal DNA comprises a coding sequence which is hereinafter referred to as a "transgene". Thus, every (or at least most) of the cells of a transgenic organism - both somatic and germ - may contain one or more copies of the transgene(s).

Transgenic organisms can be produced by many different methods. The methods are well documented in the prior art and their practice forms part of the technical repertoire of those skilled in the art. Methodological approaches commonly used are described for example in First and Haseltine (Eds.), Transgenic Animals (1991) Butterworth-Heineman MA USA.

According to one known method, the transgene is inserted into embryonic stem cells which are then injected into fertilized zygotes at a stage when only a small number of cells are present. The engineered embryonic stem cells become incorporated into the zygote, and cells derived therefrom go on to differentiate into many or all of the different cell types of the animal's body. Such cells may also include those contributing to the germline, and the progeny of such (chimaeric) animals may therefore be fully transgenic.

Other methods involve the introduction of the transgene into the pronucleus or into the fertilized or unfertilized ovum, but the invention is not limited to the method of making the transgenic organism.

According to one aspect of the present invention, therefore, there is provided a transgenic eukaryotic organism having cells containing heterologous DNA comprising a transgene encoding a negative selectable marker as herein defined. But for the selectable phenotypes arising from the transgenes, the organism may be essentially normal (i.e. not mutant for any significant character or trait with respect to the wild type and/or in that the cells exhibit normal tissue differentiation and development), the transgenes not being located, for example, such that they insertionally inactivate a gene.

According to a second aspect of the present invention there are provided eukaryotic cells (e.g. as in pending patent applications PCT/GB95/02592 and PCT/GB96/00671) containing heterologous DNA comprising a transgene encoding a negative selectable marker as herein defined. But for the selectable phenotypes arising from the transgenes, the cells may be essentially normal (i.e. not mutant for any significant character or trait with respect to the wild type and/or exhibiting normal tissue differentiation and development).

According to a third aspect of the present invention, therefore, there are provided eukaryotic immortalized cells (e.g. as in pending patent applications PCT/GB95/02591 and PCT/GB95/02497) containing heterologous DNA comprising a transgene encoding a negative selectable marker as defined herein. But for the selectable phenotypes arising from the transgenes, the cells may be essentially normal (i.e. not mutant for any significant character or trait with respect to their phenotype prior to incorporation of the selectable marker and/or

exhibiting normal tissue differentiation and development)

According to a fourth aspect of the present invention, therefore, there is provided eukaryotic cells showing any advantageous phenotype and containing heterologous DNA comprising a transgene encoding a negative selectable marker as defined herein. But for the selectable phenotypes arising from the transgenes, the cells may be essentially as before transgenesis of the selection marker (i.e. unchanged in any significant character or trait with respect to the original advantageous phenotype and/or exhibiting normal tissue differentiation and development)

As stated above, the term "essentially normal" as used herein may indicate that the cell or organism is not mutant for any significant character or trait with respect to the wild type or the phenotype prior to the insertion of the selectable marker, and/or exhibits normal tissue differentiation and development. The term "essentially normal" therefore includes transgenic organisms or cells constructed so as to provide essentially normal cells for transplantation. Since cells considered to be less immunogenic to a host, should they be implanted, can still exhibit normal tissue differentiation and development, they too would be essentially normal, as herein defined. The organism may also be essentially normal in the sense that the transgenes are resident in a silent (i.e. non-expressed region of the genome and/or in a region of the genome where transgenes do not significantly perturb the replication, segregation, organization or packing of the chromosome or its interaction with cellular components such as DNA binding proteins (including histones and regulatory elements)

The provision of transgenes encoding both a negative selectable marker as herein defined and a positive selectable marker provides great flexibility during subsequent manipulation of any eukaryotic cells of the invention in vitro. Moreover, where the invention is used to generate tissue transplants, cells of a particular type may be isolated from e.g. a transgenic animal of the invention by positive selection. The cells so isolated then may be transplanted into a non-transgenic animal to determine whether the transplant has any therapeutic effect. The transplant may be ablated by the negative selection of the invention to provide a control to determine whether the transplant was having a direct therapeutic effect.

It will beclear to those skilled in tthe art that a cell suicide mechanism could be applied to all forms of cell/tissue grafting where it is advantageous to deplete or destroy the graft, or cells from the graft. This would be of particular advantage when the cells to be depleted/destroyed undergo little or no replication as would be expected for so-called static or expanding cell populations. In terms of cell kinetics there are three populations of cells. Renewal cell populations such as those of the gut or skin, whereby progenitor and stem cells are continually dividing to produce differentiated cells which perform their required functions, and which, after a period of functioning, are lost by eg. exfoliation. Expanding cell populations do not normally divide. However, under certain conditions, such as hormone stimulation or wounding, the cells will divide. For example, the thyroid gland will increase in cell number in response to thyroid stimulating hormone. Also, liver cells will replicate in response to damage, and indeed will replace their entire cell population if eg. 75% of the liver is removed. However, in static cell populations such as neurones or cardiac muscle, cells do not divide. If a graft were to comprise static or expanding cells, then, it will be seen that the invention has a particular advantage.

The invention would allow cells from the graft, or the graft as a whole, to be depleted or destroyed readily in the absence of cell replication, by virtue of programmed cell death not

requiring replication for its initiation

The invention would be of value when any cell type or cell engineered to express a phenotype having any advantage is used, eg. cells or tissue or organs for grafting which are functionally normal but have been humanized ie. to make them immunologically more tolerable to the human host receiving the graft. The invention would also be of value when cells engineered to express proteins/factors more relevant to the human, or to the alleviation of a human medical condition, than the unmodified tissue source, are used.

The cells/organs/tissue comprising the invention would be for use as eg. allogeneic, homogeneic and xenogeneic grafts. The invention would have particular advantage in neural grafting, cardiac grafting, hepatic grafting, vascular grafting, thyroid grafting and pancreatic grafting, whether xenogeneic grafting of humanized tissue/cells, or allogeneic grafting or homogeneic grafting or other.

In another aspect, the invention provides cells or a transgenic eukaryotic organism having cells containing heterologous DNA comprising a transgene encoding a negative selectable marker of the invention as herein defined and a positive selectable marker, the organism or cells might be essentially normal as herein defined but for the selectable phenotypes arising from the transgene(s).

The cells of the invention are preferably animal cells, for example a vertebrate (e.g. a mammal, for example a rat, rabbit, pig or mouse).

The transgenic organism or cells preferably may have a genotype which is essentially normal as herein defined but for the presence of the heterologous DNA. Alternatively, the selectable cells of the invention may be derived from human or any non-human source and may not be essentially normal but be hybrids and/or express any phenotype.

In addition, that portion of the heterologous DNA which is expressed in the cells may consist of a transgene encoding a positive selectable marker and a transgene encoding a negative selectable marker as herein defined, each transgene being operably linked to an expression element or elements. The absence of expression of any other transgenically derived genetic sequences makes this a preferred transgenic organism suitable for a wide range of experimental research and cell/tissue/organ transplantation therapy requiring an effectively wild type genetic background.

A further aspect of the present invention is that the heterologous DNA which constitutes the negative selectable marker as defined herein may additionally include a multiplicity of alternative positive and/or negative selectable markers, including two or more negative selectable markers as defined herein.

At least one of the selectable markers may be operably linked to a regulatable expression element or elements, for example a tissue- or cell-specific expression element or elements. In such circumstances, each selectable marker is advantageously differentially regulated, each marker for example being linked to a different tissue- or cell-specific expression element or elements. This permits the expression of the selectable marker to be limited to a selected class of cells or tissue, or to be limited by temporal expression e.g. during a specific stage of embryogenesis, so providing e.g. for the selective culture in vitro of the selected class of cells or tissue from a mixed primary cell culture.

The present invention does not rely on the use of cells with transgenes or transgenic organisms produced by any one method: any transgenic procedure may be used in the practice of the invention. Moreover, in most circumstances, the precise nature of the selectable markers for use in the present invention is unimportant: in general, any selectable marker gene may be used so long as it additionally confers a negative selectable phenotype as herein defined on the cell.

For example, the positive selectable marker may be selected from neomycin phosphotransferase, hygromycin phosphotransferase, xanthineguanine phosphoribosyl transferase, the Herpes simplex virus type 1 thymidine kinase, adenine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase.

The negative selectable marker may, for example, be selected from any of the factors known to induce cells expressing it to undergo programmed cell death (apoptosis). Those factors might include p53, interleukin 1b converting enzyme (ICE) cysteine proteases, retinoblastoma (Rb) or mutants thereof, c-myc, bax, Fas, Fos, poly(ADP)ribose polymerase (PARP), cpp32 or YAMA or any other factor(s) or combinations thereof which induce cell death by apoptosis rather than cytotoxicity

The selectable markers are conveniently derived (e.g. by subcloning using restriction endonucleases) from any of a large number of known vectors, examples of which are described in e.g. Molecular Cloning: A laboratory Manual Second Edition Edited by Sambrook J, Fritsch and Maniatis T 1989 Cold Spring Harbour Laboratory Press) or from ATCC, or GenBank and/or EMBL databases, where vectors comprising the required selectable markers can be obtained, or information required to generate or clone the required sequences can be retrieved, and/or from the literature.

The expression elements for use in the invention may take any form so long as they can (under at least some circumstances) be made to direct and/or control the expression of the genes to which they are operably coupled. Expression elements for use in the invention may comprise transcriptional and/or translational elements, and include promoters, ribosome binding sites, enhancers and regulatory sites including activator and repressor (operator) sites. Preferred expression elements comprise promoters selected from a wide range available for use, examples of which are shown in Table 1. This Table, which is non-exhaustive, also indicates the use to which each promoter may be put in the methods of the invention described infra.

By way of example only, the expression elements for use in the invention may be selected from: promoters and/or enhancers which are specifically active in: (i) dopaminergic, serotoninergic, GABAergic, cholinergic or peptidergic neurones and sub-populations thereof; (ii) oligodendrocytes, astrocytes and sub-populations thereof; (iii) the endocrine glands, lungs, muscles, gonads, intestines, skeletal tissue or part or parts thereof; (iv) epithelial, fibroblast, fat, mast, mesenchymal or parenchymal cells; (v) particular stages of embryogenesis, and (vi) components of the blood system (e.g. T-lymphocytes, B-lymphocytes and macrophages). Alternatively they may be selected from promoters and/or enhancers which direct the transcription of genes for: (i) neurotransmitter-specific receptors; (ii) ion channels; (iii) receptors involved in ion channel gating and (iv) cytokines, growth factors and hormones.

Additionally, the expression elements for use in the invention may be inducible promoters

the transplanted cells from the nervous system, and thereby attenuates damage to otherwise normal host tissue. In addition, when the grafted or genetically engineered cells are non-dividing cells then the negative selection of the invention (which does not rely wholly or partly on cell division for its effects) is particularly advantageous. Finally, a more accurate pruning of cells numbers might be achievable if the drug required to elicit the cell death needs to be given over a short duration.

The negative selectable markers of the invention exploit the natural mechanisms by which cells are depleted in vivo, for example during normal development. These mechanisms are collectively known as programmed cell death, and are thought to underlie the process of apoptosis. Apoptosis is a fundamental mechanism for regulating cell numbers at all stages of life in multicellular organisms. During embryogenesis, for instance, various cells are selected for apoptosis: this leads to the manifestation of biological form, via either phylogenetic or morphogenetic shaping. Programmed cell death also occurs in the normal adult. For example the vertebrate haematopoietic system undergoes huge cell losses, with billions of neutrophils dying this way each day. Apoptosis also plays a major role in tissue repair and regeneration: when a cell is damaged beyond repair by e.g. ultra-violet irradiation, cell death is triggered using the cell's own suicide programme. It is thought that this altruistic act helps to maintain the organism as a whole by guarding against further replication of damaged cells which may become cancerous, thereby leading to the death of the whole organism. The control of apoptosis is currently a major component of worldwide research in cancer, since it is now believed that some cancers may result, not from an enhanced replication of tumour cells, but from a reduced ability of such cells to undergo programmed cell death.

Another advantage of using a cell's natural mechanism of programmed cell death is that macrophages and microglia react to apoptotic cells in a highly specific fashion. They engulf and digest apoptotic cells, but do not secrete inflammation-inducing signals, in contrast to their secretory profile when phagocytosing necrotic cells. Cells undergoing apoptosis rather than toxic cell death and necrosis, therefore, are not associated with a localized inflammatory response - a second unwanted contributor to the bystander effect of currently used negative selection markers.

To activate the cell disposal mechanism, apoptotic cells change their surface chemistry so that macrophages/microglia can recognize them. The alteration may be in the surface lectins or in integrins, and may vary depending on the cell type, but the final death is still via apoptosis.

The invention rests at least in part on the recognition that the natural cellular mechanism of apoptosis can be used as a safety and/or titration switch to eliminate genetically engineered cells and, in particular, cells transplanted to the central nervous system. Using such a mechanism, it is possible to induce the apoptosis and subsequent elimination of grafted cells (e.g. in the brain). This method is used advantageously, because:

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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 5/00

A2

(11) International Publication Number: WO 98/42824

(43) International Publication Date: 1 October 1998 (01.10.98)

(21) International Application Number: PCT/GB98/00654

(22) International Filing Date: 19 March 1998 (19.03.98)

(30) Priority Data:
9705744.2 20 March 1997 (20.03.97) GB

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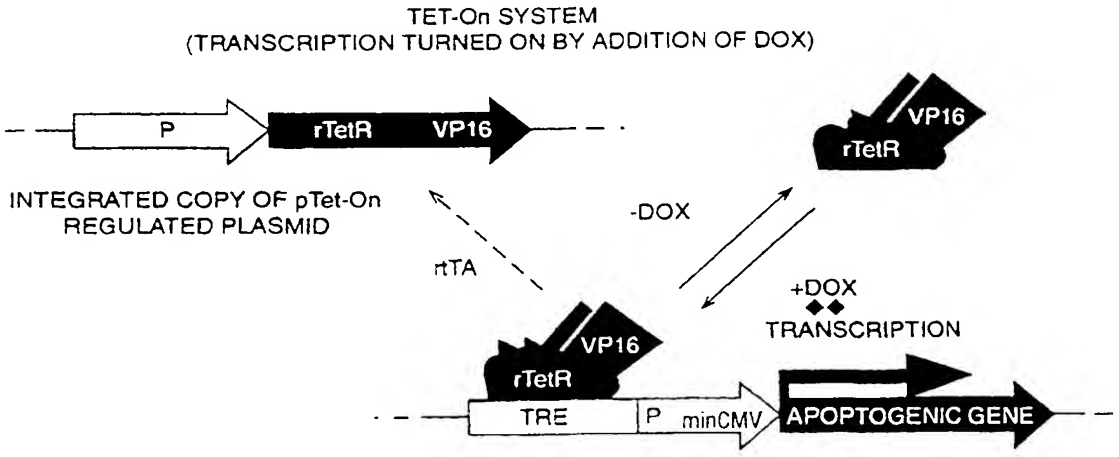
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHODS FOR SELECTING CELLS AND THEIR USES



(57) Abstract

Grafts, cells and tissues for use in transplantation, transgenic animals, methods of cell selection and various uses of such material.

which could take many forms e.g. as in Jones et al. (1991) Nucl. Acids Res. 19:6547; Woodroofe et al. (1992) DNA & Cell Biol. 11:587, Muller et al. (1992) Gene 121:253, Simson et al. (1994) Lab. Invest. 71:680; Blessing et al. (1995) Terat. Carc. Mutagen. 15:11; Shockettt al. (1995) P.N.A.S. 92:6522. Hoff et al. (1995) J. Am. Soc. Nephrol. 6:793; Maxwell et al. (1996) Gene Therap. 3:28; Walther & Stein (1996) J. Virol. 70:6054. Ho et al. (1996) Mol. Brain Res. 41:200; Delort & Capecchi (1996) Hum. Gene Therap. 7:809 or may be the Tet-offTM or Tet-onTM system currently marketed by Clontech (see Figure 1) which has been shown to be effective in both cells (Gossen et al. (1995) Science 265:1766) and transgenic organisms (Kutner et al. (1996) P.N.A.S. 193:10933) and additionally effective in brain (Mayford et al. (1996) Science 274:1675).

At least one of the selectable markers may advantageously be constitutively expressed. This ensures uniform expression of the selectable marker in every transgenic cell of the transgenic organism under all conditions, which is particularly useful where the transgenic organism is for general use as a source organism for cell/tissue culture.

Constitutive expression may be achieved for example via the use of a promoter which directs the expression of a "house-keeping" gene. A "house-keeping" gene is one which is expressed in all cell types. Their translated products are required as part of general cell metabolism or cell structure and, consequently, they are not specifically expressed in a particular cell or tissue type. House-keeping gene promoters, therefore, need to be active in a broad range of (and sometimes in all) cell types in order to ensure constitutive gene expression. When constitute promoters are used in the invention, then alternative regulation of expression of genes may be necessary. The constitutive expression could be regulated by temperature-sensitive mutants of the promoter/enhancer elements so that expression is only allowed for cells incubated within a defined temperature range. Alternatively, expression may be controlled by the requirement for factors in the medium or milieu of the cells or in the diet of the organism, by the adminsitration of factors to the organism, the absence of which would not allow active expression of genes involved in programmed cell death. Alternatively, the presence of specific factors may be required to inhibit promoter/ennancer directed expression of genes involved in programmed cell death. Control may also be achieved by regulating the biologically active form of the factor(s) required to induce apoptosis. The invention is, therefore, not limited by the method used to modulate active epxression of factors required to induce programmed cell death.

An example of a constitutively-expressed promoter useful in the present invention is that for the histocompatability complex H-2Kb class 1 promoter (Weiss et al. (1983) Nature, 301:671; Baldwin and Sharp (1987), Mol. Cell. Biol. 7:305; Kimura et al. (1986), Cell 44:261) which has been shown to express downstream coding sequences in cells generally when used as a promoter in a transgene (Jat et al (1991), P.N.A.S. 88:5096). Another example is the viral SV40 early promoter.

The promoters for use in the present invention are not restricted to those derived from mammalian cells but may also include avian- and fish-derived promoters. Additionally, virally derived promoters, some of which have biological activity in a broad range of mammalian, fish and avian cells as well as other eukaryotes, could also be used in performing the invention. Examples are the simian virus-40 derived early or late promoters, or the Long Terminal Repeats (LTR'S) of retroviruses which comprise promoter as well as enhancer elements and have the ability to promote expression of sequences under their influence in a broad range of eukaryote cells. These promoters along with supporting sequences such as ennancer

elements and other regulatory elements are well known to the man skilled in the art (see e.g. Molecular Cloning: A laboratory Manual Second Edition Edited by Sambrook J, Fritsch and Maniatis T (1989) Cold Spring Harbour Laboratory Press).

The cells or transgenic organism(s) of the invention may also contain heterologous DNA which further comprises a reporter transgene, for example β-galactosidase or luciferase. The reporter transgene may be itself operably linked to an expression element or elements which are subject to cell- or tissue-specific regulation.

Such reporter transgenes facilitate subsequent analysis of cells/tissue cultured from e.g. the transgenic organism and in particular permit the response (to for example an induced deficit in a particular class of cells/tissue) of a particular expression element or class of expression elements to be monitored in vivo or in vitro.

In another aspect, the invention provides a method of culturing cells and/or tissues in vitro, comprising the steps of: (a) providing a cell or a transgenic organism having cells containing genetic material which confers a negative selectable phenotype, as herein defined, thereon; (b) generating a primary culture of cells or a cell line or from cells and/or tissue of the transgenic organism of step (a); (c) transplanting said cells and (d) when required, selectively removing the said cells on the basis of the selectable phenotype conferred by the genetic material contained in the primary cells or the cell line or the cells of the transgenic organism.

Preferably, the cell/tissue culture method of the invention is based on the use of primary cells or cell lines or a transgenic organism having a selectable marker which incorporates the upstream selectable marker operably linked to a tissue- or cell-specific expression element or elements, whereby in step (d) a particular cell/tissue type is selectively grown on the basis of the tissue- or cell-specific expression therein of said at least one selectable marker.

This preferred method of the invention finds application for example in the selection of thyroid follicular cells from a primary (mixed cell) culture. This method may provide a primary stromal cell population of the thyroid gland in the absence of the thyroid follicular cells and constitutes a unique cell culture system useful for the study of thyroid biology and in the development of new therapeutic drugs for the treatment of thyroid diseases.

The transgenic organisms of the present invention also find application in relation to diseases involving cell loss.

Many diseases are known to be associated with specific cell and/or tissue loss. For example, in neurodegenerative disorders such as Parkinson's disease, Huntington's chorea and Alzheimer's disease one or more sub-populations of neurotransmitter-identified cells are lost during the course of the disease.

In Parkinson's disease, this loss is principally of the dopaminergic neurones of the substantia nigra region of the brain, although other cell types also decline.

In Huntington's chorea, there is a more general loss of neurones, but in this case the deficits are restricted largely to the striatum.

In Alzheimer's disease, there is a decrement in acetylcholine- serotonin- and noradrenaline-containing neurones projecting to the neo- and palaeocortex.

Other neurological diseases also stem from neural cell degeneration: the demyelination occurring in multiple sclerosis, for instance, is due to the destruction of oligodendrocytes in the brain.

The Human Immunodeficiency Virus (HIV) is known to enter cells that express the CD4 receptor and cell infection appears to lead ultimately to cell death. The loss of CD4 cells causes a catastrophic block of the entire immune system and death of the infected person.

The molecular/cellular basis of HIV induced-disease is poorly understood. This is due, at least in part, to the lack of model systems to study the pathogenesis of the disease, particularly in vivo.

The use of SIV (simian immunodeficiency virus) infected primates has been considered as a paradigm, but SIV monkeys do not acquire full-blown AIDS. In many instances, they show no symptoms at all. Alternative models that have been proposed include HIV-infected chimpanzees. Apart from the potential ethical considerations the manifestation of AIDS-like symptoms in such a model may take several years, substantially hindering research and the development of effective therapies.

Thus, animal models of the various diseases discussed above are essential as test subjects for potential pharmaceuticals and in basic clinical research. The choice of these animal models is presently very limited because of the difficulties associated with selectively destroying specific cell and/or tissue types.

Thus, according to a further aspect of the present invention there is provided a method of selectively eliminating or depleting a particular tissue or cell type in an organism, comprising the steps of: (a) providing a transgenic organism having a negative selectable marker as defined herein operably linked to an expression element (e.g. a promoter) specific for the tissue or cell type to be eliminated or depleted, and (b) administering or ceasing to administer a selective agent to the organism to eliminate or deplete that tissue or cell type on the basis of the expression therein of the negative selectable marker as defined herein. The selective agent is preferable one which induces or activates the negative selectable marker to induce programmed cell death.

The selective agent may be administered by any route. Where systemic administration is required, oral, parenteral or intravenous routes may be used. Where localized administration is required (for example where the tissue or cell-type to be eliminated is restricted to a particular organ or to a particular region of the body) targeted injection, implantation (e.g. slow release capsules) or catheterization may be used. For example, tissue in particular regions of the brain may be specifically targeted by intracerebral injection.

The method of selectively eliminating or depleting a particular tissue or cell type of the invention may be employed to provide in vivo models of diseases involving disease-related cell loss.

Accordingly, in a further aspect the present invention provides a method of modelling disease-related cell/tissue loss or atrophy comprising the steps of: (a) providing a transgenic organism having a negative selectable marker as defined herein operably linked to an expression element (e.g. a promoter) specific for the tissue or cell type which is subject to disease-related

elimination or atrophy; and (b) administering or ceasing to administer a selective agent to the organism to eliminate or deplete the tissue or cell type on the basis of the expression therein of the negative selectable marker.

The invention also provides a method (e.g. an in vitro method) of determining the effect of a deficit in a first class of cells on the characteristics of a second class of cells in an organism, the method comprising the steps of: (a) providing a transgenic organism having a first negative selectable marker as defined herein operably linked to an expression element specific for the first class of cells and either: (i) a positive selectable marker operably linked to an expression element specific for the second class of cells, or (ii) a second negative selectable marker linked to an expression element which directs the expression of the negative selectable marker in all cells of the organism except the second class of cells; (b) administering a selective agent to the organism to induce a deficit in the first class of cells on the basis of the expression therein of the negative selectable marker; (c) removing cells from the organism; and (d) selectively culturing cells of the second class from those cells removed in step (c) on the basis of; (i) the expression therein of the positive selectable marker, or (ii) the lack of expression therein of the negative selectable marker.

In another aspect the invention provides a method of screening compounds for pharmacological activity against a disease involving cell/tissue loss or atrophy, comprising the steps of: (a) providing a test model of the disease via the steps of: (i) providing a transgenic organism having a negative selectable marker as defined herein operably linked to an expression element (e.g. a promoter) specific for the tissue or cell type which is subject to disease-related elimination or atrophy, and then (ii) administering a selective agent to the organism to eliminate or deplete the tissue or cell type on the basis of the expression therein of the negative selectable marker to produce a test model; (b) administering the compound to be tested to the test model; (c) screening the compound to be tested on the basis of its effect on the test model of step (a).

The methods of the invention may be usefully applied to any disease which is associated with cell/tissue loss or atrophy. In particular, the methods of the invention find particular utility in respect to: (a) Parkinson's disease (the tissue or cell-type to be eliminated or depleted comprising dopaminergic neurones in the substantia nigra); (b) Huntington's chorea (the tissue or cell-type to be eliminated or depleted comprising neural cells of the striatum; (c) Alzheimer's disease (the tissue or cell-type to be eliminated or depleted comprising acetylcholine-, serotonin- and/or noradrenaline- neurones associated with the neo- and palaeocortex; (d) multiple sclerosis (the tissue or cell-type to be eliminated or depleted comprising brain oligodendrocytes), (e) immune disease and the cell-type to be eliminated or depleted comprises CD3, CD4 and/or CD8 cells and (f) AIDS and the cell-type to be eliminated or depleted comprises CD4 cells.

In the case of AIDS models, the method of the invention could be used to specifically deplete or eliminate CD4 cells by linking a negative selectable marker as defined herein to a CD4 cell-specific promoter (e.g. the CD4 receptor promoter). This would permit the generation of an in vivo model of AIDS by regulating the proportion of cells expressing CD4 by negative selection in vivo.

Furthermore, in the case where the transgenic animal model carries both a positive and negative selectable marker as defined herein, any residual CD4 expressing cells could later be isolated from the transgenic tissue of the animal model by positive selection in vitro for

further study. Also, the cells/tissues/organs, which may be of animal or human origin, of the invention may be usefully applied to human therapeutic areas such as Parkinson's disease, Huntington s chorea, Alzheimer's disease, stroke injury, diabetes, kidney, heart and liver dysfunction.

Examples of various promoters suitable for use in the methods of the invention described above are listed in Table 1, along with the disease(s) in which each promoter may find application.

The invention also contemplates cell/tissue cultures derived from the transgenic organisms of the invention (or produced by the cell culturing methods of the invention), and also to various therapeutic uses of the invention. The ability to trim or destroy cells/tissues/organs transplanted to e.g. humans by the method of the invention has a particular selective advantage.

The invention will now be described in more detail by way of specific examples. These examples are not intended to be taken as limiting in any way. The examples are of proposed protocols which, without wishing to be bound by any theory, it is believed could be practised (with or without modification) by those skilled in the art. The constructed sequences given below represent examples of numerous constructs that could be used to perform the invention. The invention should not be construed as being limited to their use only.

Materials

Vectors

pBabeneo plasmid vector (plasmid freely available)	Morgenstern & Land (1990) Nucl. Acids Res. 18:3587
pCI plasmid vector	Promega, 2800 Woods Hollow Rd, Madison, USA
CD2 plasmid vector	Blaese MR, NIH, Bethesda, USA (plasmid freely available) Mullen et al. (1992) P.N.A.S. 89:33; Austin & Huber (1993) Mol. Pharmacol. 43:380; Wallace et al. (1994) Cancer Res. 54:2719
TG-TKa plasmid vector (plasmid	Wallace H, Kings Buildings, University of Edinburgh, UK freely available). Wallace et al. (1991) Endocrinology 129:3217
pPBS plasmid	Morgan (1992) Nucl. Acids Res. 20:1293
Plasmid comprising wild-type p53 etc al.	Baccheti & Graham (1993) Int. J. Oncol. 3:781; Katayase et (1995) Cell Growth Diff. 6:1207; Lin et al. (1995) Cancer Surv. 25:173; Timmers & Kremer (1995) Biomed. Rev. 4:103; Hale et al. (1996) Eur. J. Biochem. 236.1; Fraser et al. (1996) Curr. Opin. Neurobiol. 6:71; Shimamura & Fisher (1996) Clin. Canc. Res. 2:435; Vaux & Strasser (1996) P.N.A.S. 93:2239; Spence et al. (1996) Expert Opin. Therap. Pats. 6.345; Gilardini et al. (1996) Clin. Immunotherap. 5:413; Moore & Thanos (1996) Prog. Neurobiol. 48:441.

Molecular Biology Reagents

Restriction endonucleases	Promega, Madison, USA
DNA modifying enzymes, ligase, CIP, T4 polymerase etc	Promega, Madison, USA
Agarose for electrophoresis	Sigma Chemical Co., St. Louis, USA
Polynucleotide kinase and and buffers	New England Biolabs Ltd., 3397 American Drive, Unit 12, Mississauga, Ontario, Canada

Construction of genes

Thyroglobulin-thymidine kinase-internal ribosomal entry site-neomycin resistance (TG-TK-a-IRES-neor)

The neomycin resistance gene (neor) was obtained from the pBabe Neo plasmin (Morgenstern & Land (1990) Nucl. Acids Res. 18:3587) by digestion with Hind III/Cla I and retrieval for the 1165 b.p. fragment containing neor gene by gel electrophoresis and the Promega Wizard PCR kit.

The pPBS plasmid (Morgan (1992) Nucl. Acids Res. 20:1293) comprising the poliovirus-derived internal ribosomal entry site sequence was digested with Hind III/Cla I. However, this could not be done simultaneously or in sequence, since the restriction sites were too close together. In order to overcome this problem, the plasmid was initially digested with Hind III and a 200 b.p. fragment of DNA containing Hind III restriction sites at both the 5' and 3' ends was inserted in order to separate the sites. The pPBS plasmid could then be digested first with Cla I and then with Hind III.

Terminal phosphate groups were removed from the Hind III/Cla I cut pPBS vector using calf intestinal phosphatase (CIP). The vector was gel-purified using a 1% agarose gel and a band containing the DNA was excised and electroeluted.

The neomycin gene was then ligated into the pPBS plasmid overnight at 15° C and the ligation reaction transformed into freshly-made MC1061 competent cells.

Positive colonies were identified by digestion of prepared plasmids with Hind III/Cla I. The neor gene and plasmid being detected electrophoretically in plasmid preparations from positive colonies. Plasmids from the positive colonies were then digested with Hinc II and Sac I (both restriction enzymes leaving digested DNA with blunt ends). The resulting Sac I/Hinc II digestion containing the IRES-neor fragment was run on a 1% electrophoresis gel and the appropriate size band was excised and the DNA electroeluted and ethanol-precipitated.

The TG-TKa plasmid (freely available from Genbank, NIH, USA accession No. J02224, Santelli et al. 1993) DNA was prepared using Promega Wizard mini preps and digested with Nar I. The ends of the plasmid were blunted using T4 Polymerase at 37° C for 1h followed by removal of the terminal phosphate groups using CIP. The CIP was inactivated by treatment of the DNA with phenol-chloroform followed by ethanol precipitation. The resulting plasmid was electrophoresed on a 1% agarose gel and the DNA was recovered and ligated with the insert

in a 1:3 molar ratio of plasmid to insert.

The ligation was incubated at 15° C overnight, and was then used to transform competent MC1061 cells. Positive colonies were selected by digestion of prepared plasmids with BamH I (the correct construct provided restriction fragments of size 3980, 1663, 3102 and 1039 b.p.).

Linearization of the plasmid was achieved by digestion of prepared plasmids with Sal I restriction enzyme. The construction is shown in Figure 2.

Thyroglobulin-wild type p53-a-IRES-neor (Tg-p53-a-IRES-neor)

This plasmid was constructed as (i)(a) above but wild type-p53 in plasmid form was obtained from Prof. J. Milner, University of York, UK and inserted in place of TK.

Cytomegalovirus-cytosine deaminase-SV40 promoter-neomycin resist-ance (CMV-CD-SV40-neor, or CD2-neor)

pCD2 plasmid (Mullen et al. (1992) P.N.A.S. 89:33) was digested with EcoR I and EcoR V, and the digest was electrophoresed on a 1% agarose gel where the 2.5 kb. fragment containing the cytosine deaminase gene, the SV40 promoter and the neomycin resistance gene was retrieved by electroelution followed by ethanol precipitation

To ensure terminal phosphate groups were present in the fragment it was treated with polynucleotide kinase.

The pCI vector was digested with EcoR 1 and Sma I (a restriction enzyme leaving the DNA with blunt ends), and the terminal phosphate groups were removed using CIP and the enzyme was inactivated with phenol/chloroform followed by ethanol precipitation. The band was then gel-purified and recovered by electroelution.

The ligation was set up containing a 3:1 molar ratio of insert to vector and was carried out at 15° C overnight. The ligation mixture was used to transform freshly-prepared MC1061 competent cells and positive colonies were selected by digestion of prepared plasmids with EcoR 1 and Hind III to provide restriction fragments of length 1868 b.p. and 5062 b.p., respectively. Linearization of the plasmid was achieved by digestion with Bgl I.

The construction is shown in Figure 3.

Cytomegalovirus-wild type p53-SV40 promoter-neomycin resistance (CMV-p53-SV40-neor)

The plasmid was constructed as in (ii)(a) above, but with p53 inserted in place of CD.

A number of other plasmid constructs can be prepared using the techniques and methods outlined in the construction of the plasmids of i(a), i(b), ii(a) and ii(b) above. All methods required to construct the plasmids are well known to the artisan of applied molecular genetics and genetic engineering.

The constructs, for example, using the Tet-On™ system available from Clontech are as follows:

1a. CMV promoter-rtetR-VP16 (this plasmid also confers neomycin resist-ance to the host cell)(see Clontech literature).
1b. TRE-Pmin CMV-wild type p53.
Both plasmids (1a and 1b) are incorporated into the same cell or to construct a transgenic organism. The cell, or cells of the organism, will express wild type p53 in the presence of e.g. doxycycline.

Choline acetyl transferase (CAT) promoter-rTetR-VP16
and 1b. are incorporated into the same cell or used to construct a transgenic organism, such that both 2 and 1b. are incorporated into cells in the transgenic organism. Cells which normally express CAT will also express wild-type p53 in the presence of e.g. doxycycline.

CAT promoter-neor-CMV promoter-rTetR-VP16
and 1b. are incorporated into the same cell or used to construct a transgenic organism, such that both 3 and 1b. are incorporated into cells in the transgenic organism. Cells will express neomycin resistance under the control of the tissue-specific CAT promoter, allowing for positive selection of CAT-expressing cells. Such cells will also express wild-type p53 in the presence of e.g. doxycycline. This provides an example of tissue-specific positive selection with negative selection, but this should not be seen as limiting since, given an understanding of the invention, the man skilled in the art could link any specific positive selection marker to create an aspect of the present invention.

The plasmids of 1b. where the wild-type p53 is replaced by ICE or Rb or mutants thereof, bas, Fas, Fos, PARP, cpp32 or YAMA or any other fator(s) which induce programmed cell death, or combinations thereof by e.g. constructs with e.g. internal ribosomal entry site separation units or multiples of the section 3 constructs.

Plasmids comprising a cell suicide gene such as p53 operatively linked to an inducible promoter as exemplified in the following publications:
Jones et al. (1991) Nucl. Acids Res. 19:6547; Woodroofe et al. (1992) DNA & Cell Biol. 11:587. Muller et al. (1992) Gene 121:263; Simson et al. (1994) Lab. Invest. 71:680; Blessing et al. (1995) Terat. Carc. Mutagen. 15:11; Shockettt al. (1995) P.N.A.S. 92:6522; Hoff et al. (1995) J. Am. Soc. Nephrol. 6.793; Maxwell et al. (1996) Gene Therap. 3:28; Waltner & Stein (1996) J. Virol. 70:6054; Ho et al. (1996) Mol. Brain Res. 41:200; Delort & Capecchi (1996) Hum. Gene Therap. 7:809 or may be the Tet-offTM or Tet-onTM system currently marketed by Clontech (see Figure 1) which has been shown to be effective in both cells (Gossen et al. (1995) Science 265:1766) and transgenic organisms (Kutner et al. (1996) P.N.A.S. 193:10933) and additionally effective in brain (Mayford et al. (1996) Science 274:1675).

Production of transgenic animals

Transgenic rats were produced by established methods (Hogan et al. (1986) Manipulating the Mouse Embryo - A Laboratory Manual, Cold Spring Harbor Lab., Cold Spring Harbor, N.Y.). In brief, approximately 2 pl of the plasmid were microinjected at a concentration of 5 µg/ml into the pronucleus of outbred Sprague-Dawley embryos. Embryos were then implanted into pseudopregnant recipients, and after identification of transgenic animals, lines were isolated and established. Lines were maintained as transgenic hemizygotes by mating hemizygous females with non-transgenic males.

Positive/negative selection of cells from transgenic animals in vitro.

Fibroblast cells

Fibroblast cultures derived from lung of adult CD2/neor, TG/TK/neor and control animals were produced and expanded by routine methods (Freshney (1987), Alan R. Liss, New York). Twenty-four hours after plating, geneticin (400 µg/ml) was added to cultures originating from both types of transgenic rats and from control rats, and replaced every three days with fresh medium. When required, cells were subcultured (1:3) to prevent them becoming confluent, again by basic culture methods (Freshney 1987). Cell counts were made manually in 20 fields chosen randomly and the values at each time point, after allowing for changes due to subculturing, were aggregated. As can be seen from Table 2, no fibroblast cells derived from control animals or the TG/TK/neor transgenic survived more than 10 days treatment with geneticin. In the absence of added geneticin, no change in cell survival from either of the transgenic animals was observed.

The effects of 5-fluorocytosine (5FC) were also determined. 5-fluorocytosine at a concentration of 100 µg/ml had no effect on fibroblast cells derived from control animals or from the TG/TK/neor transgenic. In the cells derived from the CD2/neor transgenic animal, however 94% of the orginally-plated cells died, or were non-functional (as determined by their failure to exclude trypan blue) after 10 days culture in the presence of 5FC (Table 2). By contrast, no significant difference in cell counts was found between cultures from control rats in the absence and presence of 5FC, or between controls and cultures taken from CD1/neor rats in the absence of added 5FC (Table 2).

Thyroid cells

Thyroid cultures derived from the thyroid gland of adult CD2/neor, TG/TK/neor and control animals were produced by routine methods (Freshney, 1987). Twenty-four hours after plating, geneticin (400 µg/ml) was added to cultures originating from both types of transgenic and the control rats, and replaced every three days with fresh medium. When required, cells were subcultured (1:2) to prevent their becoming confluent. Cell counts were made manually in 20 fields chosen randomly, and the values at each time point, after allowing for changes due to subculturing, were aggregated (Table 3). Ten days after the initial application of ganaticin, 10 µg/ml acycloguanosine (ACG, Sigma) was added to thyroid cells originating in the TG/TK/neor transgenic. Ten days later, cell counts were again made of 20 fields chosen at random. Results are given in Table 3. To summarize, cells derived from both types of transgenic animal survived the geneticin treatment, whereas the control cells did not. Cells derived from the TG/TK/neor transgenic did not survive ACG treatment, whereas the cells derived from the control animals did. The results were as expected in view of the specificand non-specific expression of the positive and negative selection markers, in the TG/TK/neor and CD2/neor transgenics, respectively. TG/TK/neor transgenic rat thyroid cells cultured in the absence of any added drug did not exhibit any differences intheir growth or survival compared to control thyroid cell cultures (Table 3).

Ablation of thyroid follicle cells in vivo

Adult female rats (250g) were injected intra-peritoneally with 50 mg of ACG per day for a period of 5 days. Seven days after the final injection, serum levels of T3 and T4 were measured (Amersham, UK), and found to have fallen in transgenic animals from 0.76 @ 0.05 nM to less than 0.06 nM (T3) and from 58.2 @ 3.2 nM to less than 2.5 nM (T4) (N=6).

Administration of saline to transgenic animals resulted in small but non-significant fall in T4 to 0.68 @ 0.07 nM (N=6). The thyroid glands of transgenic rats treated with ACG for 12 days had shrunk to 7% of the original weights. Histochemical analysis of these thyroid glands revealed an almost complete loss of follicular cells, with only non-follicular, perhaps calcitonin-producing cells, remaining. Administration of lower amounts of ACG per day resulted in a partial loss of T3 and T4. In most other tissues from transgenic animals, HSV-thymidine kinase activity (Brinster et al. (1981) Cell 27:223; Jamieson et al (1974) J. Gen. Virol. 24:481) was not expressed in detectable amounts. No histochemical evidence of cell loss was demonstrable in parathyroid, submaxillary or adrenal glands, nor in heart, kidney or brain.

In summary, both types of transgenic animal, or the cells therefrom, were appratently normal until application of either ACG or 5FC, as appropriate. After such application, either in vivo or in vitro, the cells upon which sensitivity had been conferred were rapidly destroyed. In addition, cells from both transgenic animals were resistant to the cytotoxic effects of geneticin, whereas cells from non-transgenic controls were completely eradicated.

Example 1: Proposed protocol for the production of a transgenic mouse bearing both positive and negative selectable markers

The herpes simplex virus (HSV) thymidine kinase gene (tk) (operably linked to the tk promoter) and the bacterial neomycin phosphotransferase (neo) gene (operably linked to the SV40 early promoter) are cloned into the appropriate cloning sites of a plasmid vector.

The plasmid vector is digested with restriction endonucleases and a fragment containing both the tk and neo selectable markers (along with the expression elements operably linked thereto) is isolated on an agarose gel.

The fragment isolated on the gel is then purified and injected into male pronuclei of fertilized one-cell mouse eggs at a concentration of 1-2 ug/ml DNA in TE buffer (10mM Tris, Ph 7.5, 0.2 mM EDTA). The eggs are those derived from a CBA x C57BL/10 mating.

The eggs which survive micro-injection are then transferred to pseudopregnant females as described e.g. in Wagner et al. (1981) P.N.A.S. 78:5016, and allowed to develop to term.

At 7-14 days of age, each pup is analysed to determine whether the transgenes are present. DNA is prepared from a section of the tail by the method described in Sambrook et al. (1989) "Molecular Cloning", Cold Spring Harbor. The presence of the neo and tk genes is determined by probing with labelled tk and neo-specific probes.

The transgenic pups so identified are mated and their offspring also analysed to check for Mendelian transfer of the transgenes.

Example 2: Proposed protocol for the selective culture of mouse thyroid follicular cells

Transgenic mice are prepared as described in Example 1, except that the neo gene is placed under the control of a thyroglobulin promoter (e.g. described by Christophe et al. (1989) Mol. Cell. Endocrinol. 64:5. Christophe et al. (1987) Ann. d'Endocrinol. 48:111; Ledent et al. (1990), PNAS. 87:6178).

The transgenic mice are sacrificed and the thyroid tissue removed and a primary culture

prepared in the presence of antibiotic G418. This antibiotic kills cells not expressing the neo gene, and results in the selective culturing within the primary (mixed cell) culture of thyroid follicular cells.

Example 3. Proposed protocol for the preparation of a rattine model of Parkinson's disease

The herpes simplex virus (HSV) thymidine kinase gene (tk) is operably linked to a promoter which is active only in dopaminergic neurones in the substantia nigra and cloned into the appropriate cloning site of a plasmid vector.

The plasmid is digested with a restriction endonuclease and a fragment containing the tk selectable marker is isolated on an agarose gel, and transgenic rats bearing the tk transgene are then prepared essentially as described in Example 1.

Ganciclovir is then administered by injection into the substantia nigra regions of the brain of the transgenic rats to specifically eliminate or deplete the dopaminergic neurones expressing the negative selectable tk marker, thus providing a rattine model of Parkinson's disease.

Example 4. Proposed protocol for the preparation of a rattine model of Alzheimer's disease

The herpes simplex virus (HSV) thymidine kinase gene (tk) is operably linked to a promoter which is active only in acetylcholine-, serotonin- and/or noradrenaline- neurones associated with the neo- and palaeocortex is cloned into the appropriate cloning site of a plasmid vector.

The plasmid is digested with a restriction endonuclease and a fragment containing the tk selectable marker is isolated on an agarose gel, and transgenic rats bearing the tk transgene are then prepared essentially as described in Example 1.

Ganciclovir is then administered by injection into the aapropriate region of the brains of the transgenic rats to specifically eliminate or deplete the acetylcholine-, serotonin- and/or noradrenaline- neurones associated with the neo- and palaeocortex expressing the negative selectable tk marker, thus providing a rattine model of Alzheimer's disease.

Table 1

Promoter	Tissue/cell-type	Application	Reference
Tyrosine hydroxylase	Catecholamin--ergic neurones	Parkinson's	1
TSH receptor	Thyroid cells	Hypothyroidism	2
B SF1 neurones	GABAergic	Epilepsy	3
Human dopamine b-hydroxylase	Noradrenaline neurones	Alzheimer's	4

Thyroglobulin	Thyroid cells	Hypothyroidism	5
Serotonin 2 receptor	Glial cells in serotonin-ergic projection areas	Neurodegenerative diseases	6
Mouse inter-leukin 4	bone cells and haem-atopoietic system	Inflammatory processes	7
C D4 receptor T-lymphocytes	CD4 expressing	AIDS	8
human choline acetyltrans-ferase	Acetylcholine neurones	Alzheimer's Motoneurone disease	9

References

1:Stachowick et al. (1994) Mol. Brain Res. 22:309
2:Ikuyama and Nawata (1994) Jap. J. Clin. Med. 52(4):962
3:Motejlek et al. (1994) J. Biol. Chem. 269:15265
4:Hoyle et al. (1994) J. Neurosci. 14:2455
5:Pichon et al. (1994) Biochem. J. 298:537
6:Ding et al. (1993) Mol. Brain Res. 20:181
7:Brunn et al. (1993) P N.A.S. 90:9707
8:Nakayama et al. (1993) Int. Immunol. 5:817
9:L. et al. (1993) Neurochem. Res. 18:271

Table 2 Survival of lung fibroblast cells derived from control and transgenic rats, and effects of various drugs

	Days in culture					
Genotype/drug	1	3	5	7	9	11
Control	100	100	100	100	100	100
TG/TK/neor	98	97	98	95	95	96
CD2/neor	92	93	92	98	105	98
Control + geneticin	9	101	85	23	5	2
TG/TK/neor + geneticin	97	105	91	27	10	3
CD2/ neor + geneticin	91	94	91	93	107	105
Control + 5FC	101	105	98	97	93	96
TG/TK/neor + 5FC	98	96	95	92	92	93
CD2/neor + 5FC	94	5	3	3	4	4

Drugs were added at day 2 in culture. Values were related to the number of cells found in control cultures without drug additions at various times after plating, and allowing for dilutions resulting from passaging. Figures are the means of three separate determinations, the

standard errors all being less than 15% of the mean.

Table 3. Survival of thyroid cells derived from control and transgenic rats, and effects of various drugs

Days in culture

Genotype/drug	1	3	5	7	9	11
Control	100	100	100	100	100	100
TG/TK/neor	91	95	93	92	101	99
CD2/neor	99	103	102	97	89	91
Control + geneticin	95	91	85	56	9	4
TG/TK/neor + geneticin	104	105	98	88	93	98
CD2/ neor + geneticin	91	94	91	93	107	105
Control + ACG	94	97	98	91	92	102
TG/TK/neor + ACG	98	38	12	10	8	7
CD2/neor + ACG	98	90	93	93	97	88

Drugs were added at day 2 in culture. Values are related to the number of cells found in control cultures without drug additions at various times after plating, and allow for dilutions resulting from passaging. Figures are the means of three separate determinations, the standard errors all being less than 15% of the mean.

CLAIMS

1. A graft (e.g. a xenograft, allograft or autograft) comprising cells containing heterologous DNA comprising a transgene encoding a negative selectable marker, wherein the negative selectable marker can be induced to activate programmed cell death or apoptosis.

2. Cells containing heterologous DNA comprising a transgene encoding a negative selectable marker, wherein the negative selectable marker can be induced to activate programmed cell death or apoptosis, e.g. for use in transplantation therapy.

3. Transplantable tissue or cells containing heterologous DNA comprising a transgene encoding a negative selectable marker, wherein the negative selectable marker can be induced to activate programmed cell death or apoptosis, e.g. for use in transplantation therapy.

4. A transgenic animal having cells containing heterologous DNA comprising a transgene encoding a negative selectable marker, wherein the negative selectable marker can be induced to activate programmed cell death or apoptosis.

5. The invention of any one of the preceding claims wherein the negative selectable marker is:
(a) a tumour suppressor gene (e.g. p53);
(b) a cell surface Fas receptor;
(c) an interleukin-1β converting enzyme (or activator thereof);
(d) c-myc or a homologue thereof;
(e) retinoblastoma gene;
(f) bax-bcl2 or a homologue thereof.

6. The invention of any one of the preceding claims wherein the cells:
(a) are essentially normal (e.g. functionally normal) but for the selectable phenotype arising from the transgene, optionally further comprising heterologous DNA comprising a transgene encoding a positive selectable marker; and/or
(b) are humanized; and/or
(c) are immunomodulated; and/or
(d) are neural, cardiac, endothelial, thyroid or pancreatic cells; and/or
(e) are derived from static cell populations, slowly replicating cell populations or expanding cell populations; and/or
(f) have a genotype which is essentially wild type but for the presence of the heterologous DNA and/or wherein that portion of the heterologous DNA which is expressed in the cells consists of the transgene encoding the negative selectable marker (and optionally a transgene encoding a positive selectable marker), the transgene(s) being operably linked to an expression element or elements.

7. The invention of any one of the preceding claims which is a vertebrate (e.g. a mammal or a non-human mammal, for example rat, rabbit, pig or mouse).

8. The invention of any one of the preceding claims wherein at least the negative selectable marker is operably linked to a regulatable expression element or elements, for example a tissue- or cell-specific expression element or elements or an inducible expression element (e.g. an inducible promoter).

9. The invention of claim 7 comprising a positive and negative selectable marker, wherein each selectable marker is differentially regulated, each marker for example being linked to a different tissue- or cell-specific expression element or elements.

10. The invention of any one of the preceding claims wherein at least one selectable marker (e.g. the positive selectable marker) is constitutively expressed.

11. The invention of any one of the preceding claims wherein the heterologous DNA further comprises a reporter transgene, for example β-galactosidase or luciferase.

12. The animal of claim 11 wherein the reporter transgene is operably linked to an expression element or elements which are subject to cell- or tissue-specific regulation.

13. The invention of any one of the preceding claims wherein:

(a) the positive selectable marker is selected from neomycin phosphotransferase, hygromycin phosphotransferase, xanthineguanine phosphoribosyl transferase, the Herpes simplex virus type 1 thymidine kinase, adenine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase, and/or

(b) the expression element is selected from:

(I) promoters and/or enhancers which are specifically active in: (i) dopaminergic, serotoninergic, GABAergic, cholinergic or peptidergic neurones and sub-populations thereof; (ii) oligodendrocytes, astrocytes and sub-populations thereof; (iii) the endocrine glands, lungs, muscles, gonads, intestines, skeletal tissue or part or parts thereof; (iv) epithelial, fibroblast, fat, mast, mesenchymal or parenchymal cells; (v) particular stages of embryogenesis, and (vi) components of the blood system (e.g. T-lymphocytes, B-lymphocytes and macrophages); or

(II) promoters and/or enhancers which direct the transcription of genes for: (i) neurotransmitter-specific receptors; (ii) ion channels; (iii) receptors involved in ion channel gating and (iv) cytokines, growth factors and hormones.

14. Tissue or cells derived or cultured from the transgenic animal of any one of claims 4-13.

15. A method of culturing cells and/or tissues in vitro, comprising the steps of:

(a) providing an animal as defined in any one of claims 4 to 13;

(b) generating a primary culture from cells and/or tissue of the animal of step (a); and

(c) selectively growing the primary culture on the basis of the selectable phenotype conferred by the genetic material contained in the cells of the animal.

16. A method according to claim 15 wherein at least one selectable marker is operably linked to a tissue- or cell-specific expression element or elements, whereby in step (c) a particular cell/tissue type is selectively grown on the basis of the tissue- or cell-specific expression therein of said at least one selectable marker, e.g. to produce a homogeneous population of a particular class of cells in primary culture.

17. A method according to claim 15 or claim 16 whereby step (c) reduces or eliminates microbial (e.g. yeast and fungal) contamination of the tissue culture.

18. Tissue or cells cultured by the method of any one of claims 15 to 17, the tissue or cells being for use e.g. as a tissue transplant, as a test subject in biochemical assays or as a source of a protein of interest.

19. Tissue or cells according to claim 14 or claim 18 for use in therapy.

20. A method of selectively eliminating or depleting a particular tissue or cell type in an organism, comprising the steps of:

(a) providing a transgenic animal according to any one of the preceding claims wherein the negative selectable marker is operably linked to an expression element (e.g. a promoter) specific for the tissue or cell type to be eliminated or depleted;

(b) eliminating or depleting that tissue or cell type on the basis of the expression therein of the negative selectable marker.

21. A method according to claim 20 for modelling disease-related cell/tissue loss or atrophy, wherein the tissue or cell type to be eliminated or depleted is that tissue or cell type which is subject to disease-related elimination or atrophy.

22. A transgenic animal according to any one of the preceding claims for use in the method of claim 20 or claim 21.

23. An animal (for example a vertebrate, e.g. a mammal) in which a particular cell/tissue is specifically eliminated or depleted, produced by the method of claim 20 or claim 21.

24. An animal according to claim 23 which is a model of disease-related cell/tissue loss or atrophy.

25. A method of screening compounds for pharmacological activity against a disease involving cell/tissue loss or atrophy, comprising the steps of:

(a) providing a test model of the disease according to the method of claim 21;

(b) administering the compound to be tested to the test model of step (a);

(c) screening the compound to be tested on the basis of its effect on the test model.

26. A method according to claim 21 or claim 25 wherein the disease is:

(a) Parkinson's disease and the tissue or cell-type to be eliminated or depleted comprises dopaminergic neurones in the substantia nigra;

(b) Huntington's chorea and the tissue or cell-type to be eliminated or depleted comprises neural cells of the striatum;

(c) Alzheimer's disease and the tissue or cell-type to be eliminated or depleted comprises acetylcholine-, serotonin- and/or noradrenaline- neurones associated with the neo- and palaeocortex;

(d) multiple sclerosis and the tissue or cell-type to be eliminated or depleted comprises brain oligodendrocytes;

(e) immune disease and the cell-type to be eliminated or depleted comprises CD3, CD4 and/or CD8 cells; and

(f) AIDS and the cell-type to be eliminated or depleted comprises CD4 cells.

27. A method (e.g. an in vitro method) of determining the effect of a deficit in a first class of cells on the characteristics of a second class of cells in an organism, the method comprising the steps of:

(a) providing a transgenic animal according to any one of the preceding claims having a first negative selectable marker operably linked to an expression element specific for the first class of cells and either: (i) a positive selectable marker operably linked to an expression

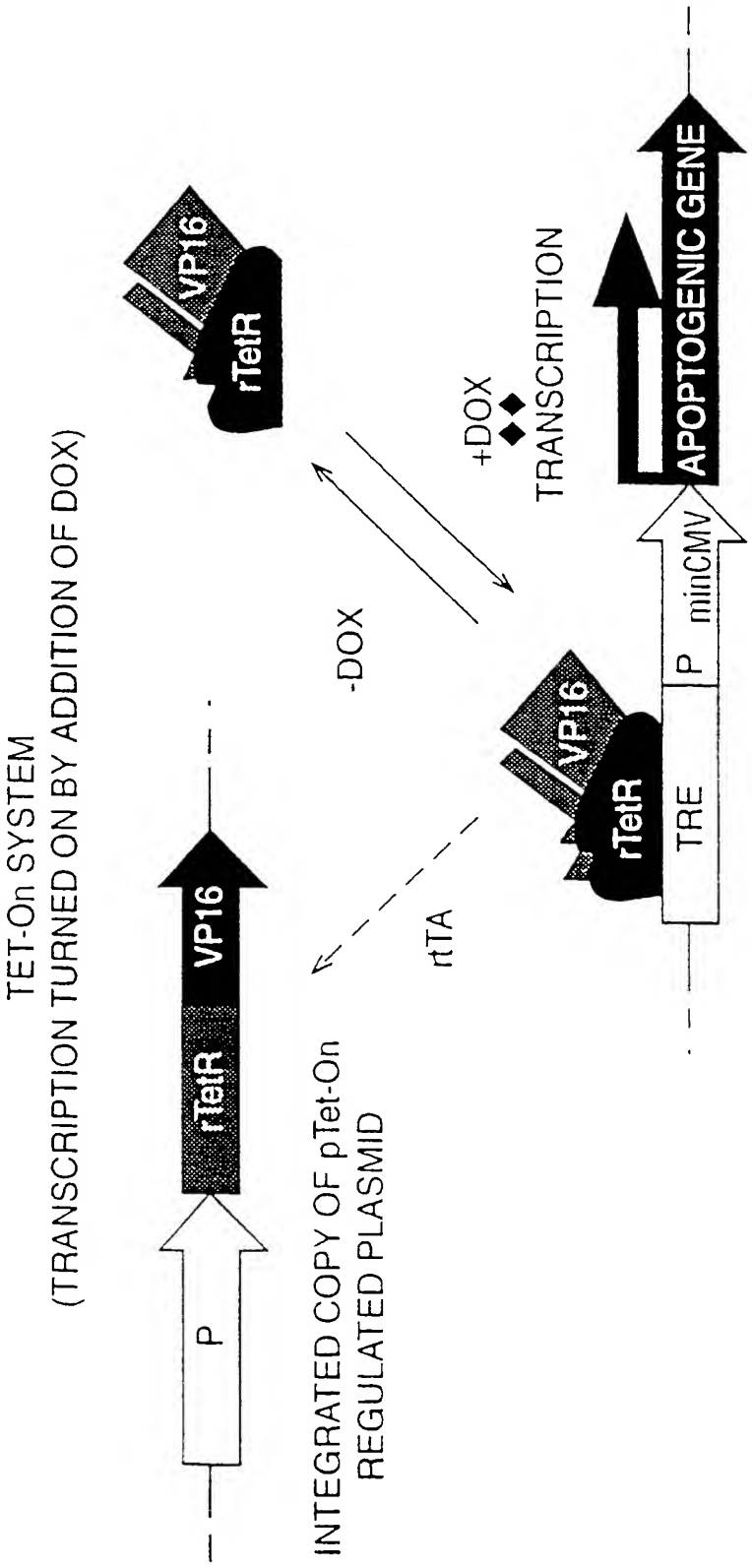
element specific for the second class of cells, or (ii) a second negative selectable marker linked to an expression element which directs the expression of the negative selectable marker in all cells of the organism except the second class of cells;

(b) administering a selective agent to the organism to induce a deficit in the first class of cells on the basis of the expression therein of the negative selectable marker;

(c) removing cells from the organism; and

(d) selectively culturing cells of the second class from those cells removed in step (c) on the basis of: (i) the expression therein of the positive selectable marker, or (ii) the lack of expression therein of the negative selectable marker.

FIG. 1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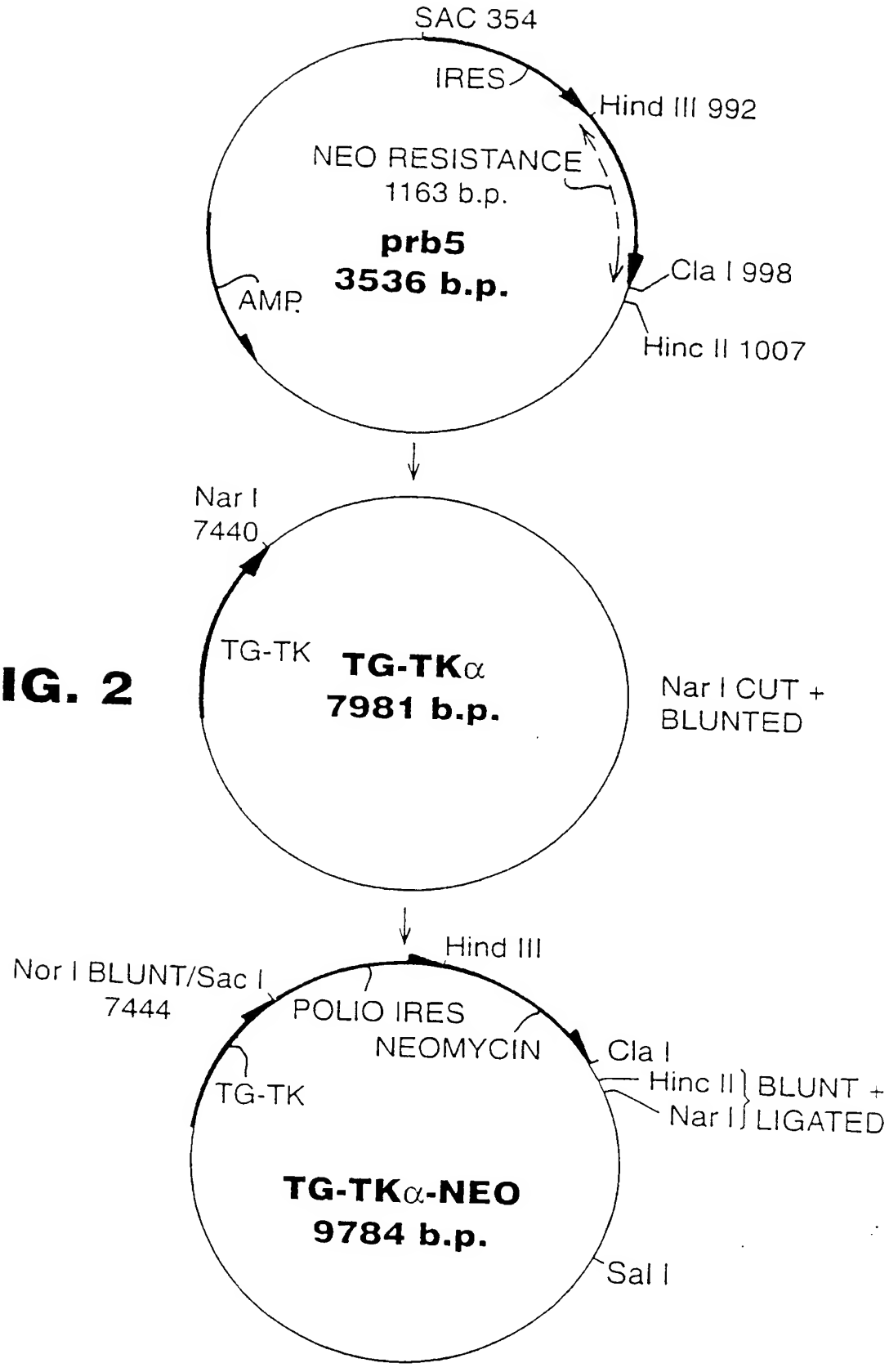
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TG-TKα
7981 b.p.
Nar I CUT +
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Nor I BLUNT/Sac I
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POLIO IRES
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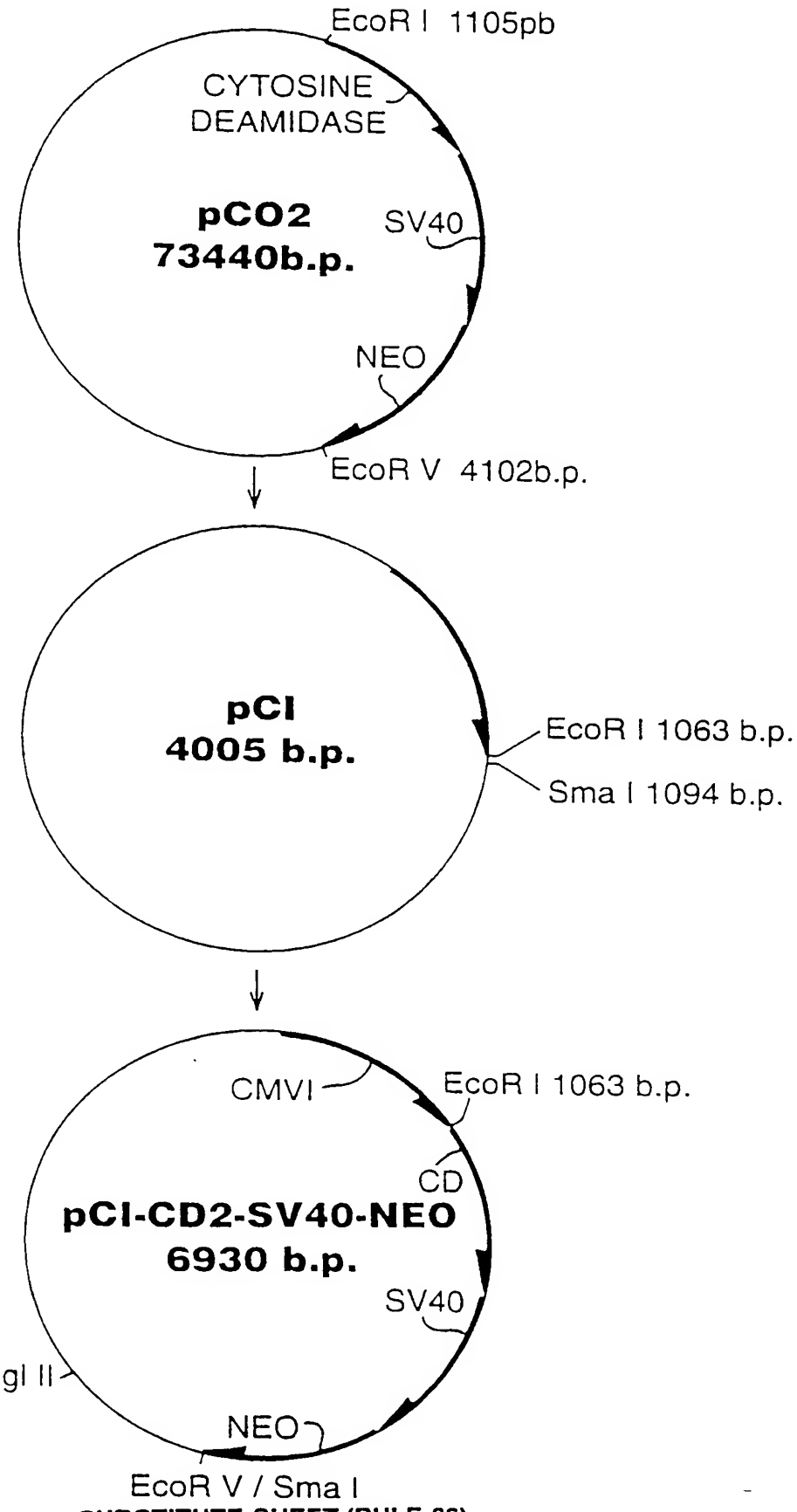
FIG. 2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pCI
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EcoR I 1063 b.p.
Sma I 1094 b.p.
CMVI
EcoR I 1063 b.p.
CD
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SV40
Bgl II
NEO
EcoR V / Sma I

FIG. 3

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 98/00654

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/06 A01K67/027 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K A01K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2 294 945 A (STRINGER BRADLEY MICHAEL JOHN) 15 May 1996 see abstract see page 6, line 5 - line 10 see page 10, line 6 - page 23, line 20 ---	1-3,5-8
X	WO 97 07828 A (UNIV CALIFORNIA) 6 March 1997 see abstract see page 4, line 18 - page 7, line 19 see page 12, line 10 - page 19, line 30 ---	1-3,5
X,P	WO 97 45142 A (GENETIC THERAPY INC) 4 December 1997 see abstract see page 6 - page 17 * examples, claims * --- -/--	1-3,6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 1998	15/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/00654

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 97 18307 A (SANDOZ AG ;BUEHLER THOMAS (CH); SANDOZ LTD (CH); SANDOZ AG (DE)) 22 May 1997 see abstract see page 1, line 1 - line 30	1
A	WO 96 14420 A (CANCER RES CAMPAIGN TECH ;AGRICULTURAL & FOOD RES (GB)) 17 May 1996 see abstract see page 3, line 15 - page 4, line 17	4,20

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/00654

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 27 (partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/ GB 98 /00654

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 27 (partially)

Claim 27 (an in vitro method of determining the effect of a deficit of a first class of cells on the characteristics of a second class) has neither reference to any of the precedent claims nor to the specific methods and embodiments of the application. These are namely the selectable positive marker and the induced apoptosis as negative marker and the targeted cells and tissues.
Therefore the teachings of claim 27 go broadly beyond the embodiments of the proposed invention.
Nonetheless, a search for claim 27 has been partially performed and has been limited according to the genes, cells and methods as defined previously in the application.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[illegible]tional Application No
PCT/GB 98/00654

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 0861092 A	02-09-1998
WO 9745142 A	04-12-1997	AU 3079697 A	05-01-1998
WO 9718307 A	22-05-1997	AU 7684896 A	05-06-1997
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